Rapid Communication

Streptomyces venezuelae TX-TL – a next generation cell-free synthetic biology tool

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Abbreviations: aTC, Anhydrotetracycline; **sfGFP**, Superfolder green fluorescence protein; **TX-TL**, *in vitro* transcription-translation

Abstract

Streptomyces venezuelae is a promising chassis in synthetic biology for fine chemical and secondary metabolite pathway engineering. The potential of *S. venezuelae* could be further realized by expanding its capability with the introduction of its own *in vitro* transcription-translation (TX-TL) system. TX-TL is a fast and expanding technology for bottom-up design of complex gene expression tools, biosensors and protein manufacturing. Herein, we introduce a *S. venezuelae* TX-TL platform by reporting a streamlined protocol for cell-extract preparation, demonstrating high-yield synthesis of a codon-optimized sfGFP reporter and the prototyping of a synthetic tetracycline-inducible promoter in *S. venezuelae* TX-TL based on the TetO-TetR repressor system. The aim of this system is to provide a host for the homologous production of exotic enzymes from Actinobacteria secondary metabolism *in vitro*. As an example, we demonstrate the soluble synthesis of a selection of enzymes (12-70 kDa) from the *Streptomyces rimosus* oxytetracycline pathway.

1 Introduction

Streptomyces belongs to the high G+C (%) Actinomycetes soil bacteria and represents the leading source of natural antibiotics such as streptomycin and tetracycline [1]. Recently, *Streptomyces venezuelae*, the chloramphenicol producer, has been adopted by synthetic biology for its use in metabolic engineering [2], since it is relatively well characterized, has strong promoter tools and genome engineering plasmids for integration [3–5]. Whilst it is not as characterized as *Streptomyces coelicolor* A3(2), in contrast, *S. venezuelae* provides significant advantages such as a fast growth (~ T_D = 40 min) and no aggregation during liquid culture [3]. One potential route for the further development of *S. venezuelae* and the characterization of its genetic parts is the introduction of an *in vitro* transcription-translation system (TX-TL). TX-TL has recently been developed as a highly adaptable tool for bottom-up synthetic biology and is based on a whole-cell extract [6–9] to synthesize recombinant proteins from the chemical building blocks of life.

One potential new application for TX-TL is the direct assembly of natural products from biosynthetic genes, as recently pioneered in *E. coli* TX-TL for the co-synthesis of two large (> 100 kDa) non-ribosomal peptide synthetases [10]. Indeed, for expression of genes from *Streptomyces* species, *E. coli* may not be the ideal host chassis in all cases - e.g. poor codon usage, solubility issues, post-translational modification [11] or an absence of exotic precursors, such as coenzyme F_{420} [12]. Moreover, utilizing a host homologous to the chosen pathway has previously proved successful for acquiring soluble and active pathway enzymes [13], whereas *E. coli* accumulated only inclusion bodies. Another caveat to the use of *E. coli* is the potential inhibition of TX-TL machinery if the target products

possess antimicrobial activities, whereas many *Streptomyces* species are known to provide their own resistance strategies to a variety of antimicrobials [14].

There is past evidence to suggest that a range of *Streptomyces* species are suitable for TX-TL [15,16], however, it is unclear from recent literature what is the true productivity of a *Streptomyces* TX-TL system. TX-TL can provide a tool to rapidly prototype the cellular machinery of synthetic biology hosts [17]. Herein, we provide evidence for the development of a high-activity *S. venezuelae* TX-TL system utilizing the *kasOp** promoter as a standard for cell-extract optimization [18]. In summary, we demonstrate high-yield synthesis of up to 1.3 μM superfolder GFP (sfGFP), prototype a TetR-TetO gene expression tool [19] and synthesise a selection of enzymes from the *S. rimosus* (ATCC 10970) oxytetracycline pathway [20].

2 Materials and methods

Materials and methods section is provided in Supporting information

3 Results

3.1 Optimising a high-activity *S. venezuelae* cell-extract

A general protocol for *Streptomyces* TX-TL was previously developed by Hopwood, Bibb and colleagues [21]. Significantly, a number of costly and therefore undesirable components are present in this original protocol, such as *Staphylococcal* nuclease and pyruvate kinase. To try a different low-cost strategy, we prepared a *S. venezuelae* cell-extract using the original *Streptomyces* method and tested its activity with a 3-phosphoglyceric acid (3-PGA) energy buffer derived from *E. coli* TX-TL [22]. Cell-extracts were tested for activity using sfGFP reporter coupled to a high-activity *kasOp** promoter.

The process of cell-extract preparation can be divided into five stages in the order of (1) cell-growth, (2) washing, (3) sonication, (4) run-off and (5) dialysis. We merged the *Streptomyces* method for stages (1-2) with the *E. coli* TX-TL methodology for stages (3-5). This new protocol provided a significant baseline level of sfGFP fluorescence (154 nM), whereas by following the separate protocols on their own, only trace levels of sfGFP fluorescence (~5 nM) were observed (data not shown). Next, by focusing on the preparation of the cell-extract and the reaction conditions, key variables (Fig. 1A-F) were optimised such as cell-lysis by sonication, run-off, dialysis and the concentration of TX-TL reaction components including polyethylene glycol (PEG), Mg-glutamate and K-glutamate. Each of these single components was varied and assessed during two rounds of parameter optimization to establish a streamlined protocol for *S. venezuelae* TX-TL, providing a maximum yield of 1.31 μM sfGFP (36 μg mL^{-1}), which demonstrated an 8.5-fold increase over the original base levels. In brief, a significant gain in activity was observed by varying the levels of the molecular crowding agent PEG and Mg-/K-glutamate salt (Fig. 1B, C),

whilst the sonication duration did not appear to alter the activity. Dialysis in S30-SC buffer was also found to reduce cell-extract activity by 18 %.

3.2 TX-TL protein synthesis requires 20-40 nM of DNA for translation saturation

The *S. venezuelae* cell-extracts were active for up to 4 hours of TX-TL batch synthesis. Interestingly, the signal intensity of sfGFP in both real-time TX-TL fluorescence and Western blot end-point samples demonstrated a proportional increase in sfGFP production with plasmid DNA concentration, which saturate between 20-40 nM of DNA in three independent batches (Fig. 2A, 2B). For comparison, in *E. coli* Rosetta TX-TL extracts, saturation requires 10-15 nM [17]. In addition, for *S. venezuelae* TX-TL, the fluorescence signal for sfGFP was observed to decay after approximately 4 hours of incubation. This also occurred with incubations pre-spiked with purified sfGFP (Supporting information, Fig. S2). The signal decay was suspected to be due to host proteases, however, a Western blot prepared with anti-GFP primary antibody confirmed that only single full-length sfGFP (27 kD) species was present in the extracts (Fig. 2C). A possible explanation for this fluorescence decay is non-specific unfolding or aggregation of sfGFP within the cell-extract.

3.3 TX-TL synthesis of the OxyB, -C, -D, -J, –K and –T enzymes

To test the ability of *S. venezuelae* TX-TL to synthesise proteins from secondary metabolism, a selection of genes (*oxyB*, *-C*, *-D*, *-J*, *-K* and *–T*) from the *S. rimosus* oxytetracycline pathway [20] were assembled by Golden Gate with a T7 promoter, strong RBS and C-terminal His_6-tag. In addition, a T7-driven sfGFP (-/+ His_6-tag) was used as a positive control, with T7 RNA polymerase added to the *S. venezuelae* extracts to drive mRNA synthesis. Interestingly, for all of the oxytetracycline enzymes OxyB –C, D, -J, -K, -T

and sfGFP, these were detected by Western blotting as full-length His_6-tagged proteins (Fig. 2D), ranging in size from 12 kDa (OxyC) to 70 kDa (OxyD). This provided an indication of the potential of *S. venezuelae* TX-TL to synthesise high G+C (%) genes from secondary metabolism. In comparison, with *E. coli* TX-TL, although higher yields (~2-10 μM) of sfGFP and the OxyB, -C, -J, -K and –T proteins were obtained, the OxyD protein could not be detected. For further information please refer to Supporting information Fig. S3.

3.4 Inducible gene expression with the TetR system

Utilising the Tn10-derived TetR gene expression tool [19], we introduced a *tetO* operator site immediately downstream of the *kasOp** promoter. By assembling this synthetic promoter with sfGFP, an anhydrotetracycline (aTC) inducible gene expression tool was rapidly prototyped in *S. venezuelae* TX-TL by utilising purified cognate TetR repressor, aTC and the TX-TL reaction components (Fig. 3). Firstly, 20 nM *kasOp*-tetO* synthetic promoter produces 3.5-fold less sfGFP in comparison to the equivalent *kasOp**-sfGFP control plasmid (20 nM) lacking the *tetO* operator. Additionally, gene expression from the *kasOp**-sfGFP plasmid is unaltered with either TetR (5 μM) or 1 μM aTC, whilst a 26% decrease in signal is observed with 10 μM aTC. However, in the presence of the *tetO* operator coupled to the *kasOp** promoter and sfGFP, a clear switch-off in gene expression is observed by titrating an increasing concentration of TetR (1-5 μM), which was recovered by the presence of aTC (1-10 μM).

3.5 Concluding remarks

Herein, we have developed a *S. venezuelae* TX-TL system as a new tool for synthetic biology. This system demonstrates high-yield synthesis of sfGFP (up to 1.3 μM) and a

range of enzymes from the oxytetracycline pathway, using a simple and cost-efficient protocol for extract preparation. The development of a *S. venezuelae* TX-TL system potentially provides a fast route to obtaining enzymes from *Streptomyces* secondary metabolism using a homologous host for protein folding. Whilst each protein target is unique, we will investigate this tool for the synthesis of specialised enzymes that require post-translational modification [10,11] or exotic precursors for protein folding, such as coenzyme F_{420} [12]. We will now focus on enhancing this initial *S. venezuelae* TX-TL platform, prototype gene circuits and investigate its use for the characterisation of cryptic gene clusters located within the Actinomycetes bacteria [23,24].

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Conflict of interest
The authors declare no financial or commercial conflict of interest.

4 References

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Figure 1. A protocol for *S. venezuelae* TX-TL. (**A**) Schematic overview of cell-extract preparation. Optimisation of (**B**) Mg-/K-glutamate, (**C**) PEG, (**D**) run-off reaction, (**E**) sonication and (**F**) dialysis conditions, with 10 nM of *kasOp**-sfGFP plasmid. Errors bars (standard deviation) are representative of three biological and three technical repeats. (**G**) Batch variation between cell-extracts with 40 nM of *kasOp**-sfGFP plasmid.

Figure 2. Saturation of *S. venezuelae* TX-TL occurs at 20-40 nM DNA. (**A**) Time-course reaction with increasing DNA concentration. (**B**) Saturation curve of end-point samples with Extracts A-C. Errors bars (standard deviation) are representative of three technical repeats. (**C**) Western blot of end-point TX-TL samples with mouse anti-GFP primary antibody. Lane abbreviations: M, PageRuler Plus (ThermoFisher) and purified His_6-sfGFP was used as a positive control (29 kDa). (**D**) Western blot of *S. venezuelae* and *E. coli* TX-TL of sfGFP and oxytetracycline enzymes. Red star (*) indicates negative synthesis of OxyD in *E. coli* TX-TL. Positive bands are individually cropped, with the original blots and SDS-PAGE gels shown in Supporting Information Fig. S3.

Figure 3. Synthetic TetO/TetR gene expression in *S. venezuelae* TX-TL. (**A**) Plasmid design of the *kasOp*-tetO* synthetic promoter. (**B**) Purified TetR and aTC was spiked into *S. venezuelae* TX-TL to modulate gene expression. (**C**) End-point readings of sfGFP with varied TetR and aTC concentrations in combination with the *kasOp*-tetO*-sfGFP plasmid. aTC inhibition with (**D**) *kasOp**-sfGFP and (**E**) *kasOp*-tetO*-sfGFP. (**F**) Repression with TetR and (**G**) release with 1 μM aTC (non-inhibitory level). Errors bars (standard deviation) are representative of three technical repeats.